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DNA Extracted from Stained Sputum Smears Can Be Used in the MTBDR*plus* Assay▽

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We examined the feasibility of using DNA extracted from stained sputum smears for the detection of rifampin and isoniazid resistance with the commercial MTBDR*plus* assay from Hain Lifescience GmbH, Nehren, Germany. Overall sensitivity was initially low (70.0%) but increased to 96.7% when a multiplex PCR preamplification step was added. We then tested stored *Mycobacterium tuberculosis*-positive stained smears prepared from 297 patients' sputum samples. Species identification and drug susceptibility testing (DST) had been performed at the Institut Pasteur de Madagascar. Overall, the performance of the MTBDR*plus* assay applied to slide DNA was similar to that obtained in other studies with DNA extracted from clinical specimens. With the ready availability of stained smears in routine diagnostic laboratories and their easy transport and storage at room temperature, this approach should be useful for optimizing the treatment of multidrug-resistant tuberculosis and for conducting resistance surveys aimed at identifying hot-spot regions and breaking chains of transmission.

Multidrug-resistant tuberculosis (MDR-TB) poses a formidable worldwide challenge for control programs, because of complex diagnostic and treatment issues. Less than 5% of MDR-TB patients are currently being diagnosed, owing to a severe lack of laboratory capacity. Alarming increases in MDR-TB, the emergence of extensively drug resistant TB (XDR-TB), potential institutional transmission, and the rapid progression of HIV-coinfected MDR-TB and XDR-TB patients call for rapid screening methods (25). Novel technologies for rapid detection of anti-TB drug resistance have therefore become a priority in TB research and development, and molecular line-probe assays have already been developed for rapid detection of resistance to rifampin (RIF), isoniazid (INH), fluoroquinolones, and injectable drugs (9, 25).

The MTBDR*plus* assay (Hain Lifescience GmbH, Nehren, Germany) has estimated sensitivities of 91.7% to 100% and 73% to 100% for the detection of RIF and INH resistance, respectively, in clinical strains (1–3, 6, 8, 10, 11, 13, 15, 17). However, because of the need for trained staff, specific equipment, and dedicated infrastructure, such tests are currently restricted to specialized laboratories. Even when these tests are available, detection of resistance in developing countries is often restricted by transport and storage of sputum samples from remote locations.

It has been shown that *Mycobacterium tuberculosis* complex DNA present in Ziehl-Neelsen- or auramine-stained smears can be extracted and used in molecular tests (7, 19, 21–24). In this study, we show that the MTBDR*plus* assay can be applied to DNA extracted from microscopy-positive stained sputum smears from patients with relapse, treatment failure, and newly diagnosed TB.

MATERIALS AND METHODS

Stored stained slides. We selected 297 stored smear-positive stained slides prepared from TB patients' sputum samples between January 2003 and March 2010. Species identification and drug susceptibility testing (DST) had been performed at the Institut Pasteur de Madagascar. All the slides bore laboratory identification numbers inscribed with a diamond or in pencil and had been stored in the laboratory at room temperature. Results of direct examination and culture were available in the lab records. Conventional DST showed that 53 of the strains present in the 297 sputum samples were MDR, 10 were RIF monoresistant, 36 were INH monoresistant, and 198 were fully susceptible.

Sputum specimens. Thirty sputum specimens were randomly chosen from among stored samples corresponding to the slides included in the study.

Conventional DST. The standard proportion method (4) was used for INH and RIF susceptibility testing on Lowenstein-Jensen medium with 0.2 μg/ml INH (reference number I-3377; Sigma) and 40 μg/ml RIF (reference number R-8626; Sigma). The critical proportion of resistant bacilli was 1% for both drugs. A strain was considered MDR when it was resistant to both INH and RIF.

DNA extraction from sputum. DNA was extracted from sputum as recommended by the MTBDR*plus* assay manufacturer. Briefly, 500 μl of *N*-acetyl-L-cysteine–NaOH-decontaminated sputum (12) was centrifuged at 10,000 × *g* for 15 min. The supernatant was discarded, and the pellet was resuspended in 100 μl of distilled water by vortexing. Finally, the specimen was boiled for 20 min and incubated in an ultrasound bath at room temperature for 15 min.

DNA extraction from slides. The Chelex method was used for DNA extraction from the glass slides, as previously described (23). Mineral oil was removed with xylene, and the smears were scraped off the slides after adding 25 μl of sterile distilled water before transfer to microtubes. Chelex suspension (75 μl) containing 5% Chelex-100, 0.01% lauryl sulfate, 1% Nonidet P-40, and 1% Tween 20 was added to the samples and thoroughly mixed prior to incubation in boiling water for 30 min. The samples were then centrifuged for 15 min at 13,000 × *g*, and the supernatants were transferred to fresh microcentrifuge tubes and stored at −20°C until use.

Multiplex PCR preamplification. In order to increase the sensitivity of the MTBDR*plus* assay applied directly to slide DNA extracts, a 847-bp region of the *rpoB* gene, a 930-bp region of the *katG* gene, a 752-bp region of the *inhA* gene, and a 852-bp region of the 23S rRNA gene were amplified by multiplex PCR using the following respective primers: rpo1 (5′-CTCAAGGCGCTGGGCTGGAC-3′) and rpo2 (5′-CCACCTTGCGGTACGGCGTT-3′), kat1 (5′-GCAACTGCGCGCTGGAATCG-3′) and kat2 (5′-CCGCCCATGCGGTCGAAACT-3′),

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TABLE 1. MTBDR*plus* assay results compared to conventional DST results for the 254 DNA samples extracted from slides

MTBDR*plus* assay result	No. (%) of strains with the indicated conventional DST result					
	RIF		INH		MDR	
	Resistant (n = 48)	Susceptible (n = 206)	Resistant (n = 69)	Susceptible (n = 185)	MDR (n = 40)	Non-MDR (n = 214)
Resistant[a]	47 (97.92)	4 (1.94)	55 (79.71)	4 (2.16)	33 (82.5)	4 (1.87)
Susceptible[b]	1 (2.08)	202 (98.06)	14 (20.29)	181 (97.84)	7 (17.5)	210 (98.13)

[a] MDR for MDR strains on conventional DST method.
[b] Non-MDR for MDR strains on conventional DST method.

inh1 (5′-GCGGCGGTTGGTGTCGATGA-3′) and inh2 (5′-GAACGCCCCGG TGAGGTTGG-3′), and 23S1 (5′-GCAGGCATGAGTAGCGACAAGG-3′) and 23S2 (5′-CCATTCGTGCAGGTCGGAACTT-3′). *Phusion* HF buffer (Finnzymes), 200 nM deoxynucleoside triphosphate, 500 nM each primer, 1 U of *Phusion* DNA polymerase (Finnzymes), and 5 μl of DNA template (from slides) in a final volume of 50 μl were used in the following 2-step PCR protocol in an automated thermal cycler (Multigene; Labnet): 30 s of denaturation at 98°C, 45 cycles with one denaturation step at 98°C for 10 s and one annealing and elongation step at 72°C for 30 s, and a final extension step at 72°C for 10 min.

GenoType MTBDR*plus* test. The GenoType MTBDR*plus* test was performed according to the manufacturer's instructions (Hain Lifescience GmbH, Nehren, Germany). For the amplification process, 5 μl of the DNA extract or preamplification product was used. The PCR protocol was modified by increasing the number of cycles from 30 to 45 when using preamplified DNA or DNA directly extracted from stained slides. Hybridization and detection were performed according to the manufacturer's instructions. The MTBDR*plus* assay strip contains 27 reaction zones, of which 21 correspond to probes for mutations and 6 correspond to control probes for internal validation. The six control probes include a conjugate control, an amplification control, a *M. tuberculosis* complex-specific control (TUB), an *rpoB* amplification control, a *katG* amplification control, and an *inhA* amplification control. Eight wild-type (WT) *rpoB* probes encoding amino acids 505 to 533 and four probes for common mutations are used to detect RIF resistance. The probes used for INH resistance detection comprise one for a WT *katG315* region, two probes for mutations in the highly resistant *katG* gene, two probes specific for WT regions, and four probes for the *inhA* gene mutation, which is associated with low-level resistance. The absence of at least one of the wild-type bands or the presence of bands indicating a mutation in a drug resistance-related gene implies that the strain is resistant to the corresponding antibiotic. When all the wild-type probes for a given gene are positive and there is no detectable mutation within the region examined, the sample is considered susceptible to the corresponding antibiotic. A result is considered valid when all six control bands develop properly on the strip. Otherwise, the result is considered invalid and the assay must be repeated. The person who read and recorded the MTBDR*plus* bands was blinded to the results of conventional DST.

For samples presenting some discrepancies between the susceptibility results of the MTBDR*plus* assay and preamplification product along with conventional DST, the MTBDR*plus* assay was repeated with the corresponding DNA extracted from sputum samples without the preamplification step according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed with R software (R Development Core Team, R: a language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2010; http://www.R-project.org).

Sensitivity and specificity were calculated with standard methods. Sensitivity was defined as the ability to detect resistance, and specificity was defined as the ability to detect susceptibility. Comparisons were based on the McNemar chi-square test. Statistical significance was assumed at a P value of <0.05. Agreement between the two methods was calculated with the kappa coefficient (κ). Common interpretations for the kappa statistic were as follows: <0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, substantial agreement; >0.8, almost perfect agreement. McNemar's test was used to test for bias. Nonsignificance in McNemar's test indicates that two proportions do not differ and that the kappa statistic is a valid measure of agreement.

RESULTS

Results of GenoType MTBDR*plus* assay with template DNA extracted from microscope slides with or without preamplification. First, we tested the feasibility of the method by using template DNA extracted from 31 randomly chosen smear-positive slides for the MTBDR*plus* assay. Smear positivity ranged from 1+ to 3+, on the basis of IUATLD/WHO guidelines (18). No correlation between the quantity of acid-fast bacteria and the band intensities was observed. The results were valid for 22/31 (70.9%) of the slides. In an attempt to improve the sensitivity of the method, we retested the same 31 DNA extracts after preamplification of the target genes by multiplex PCR. This nested PCR step increased the rate of valid results to 30/31 (96.7%) and was therefore included in subsequent analyses.

Results of GenoType MTBDR*plus* assay with template DNA extracted from microscope slides and from the corresponding sputum samples. To determine whether identical resistance results were obtained with DNA extracted from sputum and slides, 30 paired smear-positive slides and sputum samples were randomly chosen for analysis. Sputum DNA was used directly in the MTBDR*plus* assay, while slide DNA was preamplified by multiplex PCR. Identical results were obtained for all 30 paired specimens (100%).

Evaluation of MTBDR*plus* assay with template DNA extracted from slides. DNA from 297 smear-positive slides with known conventional DST results was extracted, preamplified, and tested with the MTBDR*plus* assay. Valid results were obtained for 254 slides (85.6%). Thirty-three slides (11%) gave invalid results due only to failure of the TUB control, while 10 slides gave invalid results due to failure of the *rpoB*, *inhA*, and/or *katG* control. Table 1 compares the distribution of the MTBDR*plus* and conventional DST results. For all discordant results, the MTBDR*plus* assay was repeated with the corresponding DNA extracted from sputum samples without the preamplification step. Identical results were obtained for all paired samples. The MTBDR*plus* and DST results for RIF and INH resistance and MDR agreed for 98.03% (249/254; $\kappa = 0.937$; 95% confidence interval [CI], 0.883 to 0.992; $P < 0.0001$), 92.91% (236/254; $\kappa = 0.812$; 95% CI, 0.73 to 0.895; $P < 0.0001$), and 95.67% (243/254; $\kappa = 0.832$; 95% CI, 0.735 to 0.928; $P < 0.0001$) of the cases, respectively.

With respect to the DST results, the sensitivities of the MTBDR*plus* assay for RIF and INH resistance and MDR were 97.92% (47/48; 95% CI, 88.93 to 99.95%), 79.71% (55/69; 95% CI, 68.31 to 88.44%), and 82.5% (33/40; 95% CI, 67.22 to 92.66%), respectively, and the specificities were 98.06% (202/

TABLE 2. Gene mutations conferring RIF resistance identified by the MTBDR*plus* assay

Conventional DST result[b] (no. of slides)	No. (%) of slides	MTBDR*plus* assay	
		RIF pattern[a] (*rpoB*)	Mutation or mutated codon (*rpoB*)
RIF^r (47)	27 (52.94)	ΔDWT8, MUT3	S531L
	6 (11.76)	ΔWT8	530–533
	2 (3.92)	WT, MUT3	S531L
	2 (3.92)	ΔWT7, MUT2A	H526Y
	1 (1.96)	WT, MUT2A	H526Y
	1 (1.96)	WT, MUT2A, MUT3	H526Y and S531L
	3 (5.88)	ΔWT7	526–529
	2 (3.92)	ΔWT2	510–513
	3 (1.96)	ΔWT2, ΔWT3	510–517
RIF^s (4)	2 (3.92)	ΔWT2	510–513
	1 (1.96)	ΔWT8, MUT3	S531L
	1 (1.96)	WT, MUT3	S351L

[a] WT, wild-type pattern; Δ, missing bands; MUT, mutation.
[b] RIF^r, rifampin resistant; RIF^s, rifampin susceptible.

206; 95% CI, 95.1 to 99.47%), 97.84% (181/185; 95% CI, 94.56 to 99.41%), and 98.13% (210/214; 95% CI, 95.28 to 99.49%), respectively.

The most frequent mutations identified by the MTBDR*plus* assay were *rpoB* S531L (62.74%) and the unidentified mutation in the region from codons 530 to 533 (11.76%) (Table 2). The main mutations for INH resistance were *katG* S315T (74.57%) and *inhA* C15T (22.03%) (Table 3).

DISCUSSION

This study demonstrates the feasibility of using DNA extracted from positive stained sputum smears for resistance testing with the MTBDR*plus* assay. The inclusion of a multiplex PCR preamplification step significantly increased the overall sensitivity from 70.9% to 96.7% ($P < 0.05$), a sensitivity similar to that reported elsewhere with sputum specimens (78.5% to 100%) (3, 8, 13–15, 17). Likewise, performance for the detection of RIF resistance, INH resistance, and MDR was similar to that reported in the literature (1–3, 6, 8, 10, 11, 13, 15, 17) for sputum and/or clinical specimens. When RIF resistance (RIF^r) was considered a marker of MDR (16, 20), sensitivity increased to 100% (40/40).

Discordant results between the MTBDR*plus* assays with slide extracted DNA and conventional DST were observed (Table 1). When the MTBDR*plus* assay was repeated with DNA extracted from sputum samples according to the manufacturer's instructions, we found the same results obtained with preamplified slide extracted DNA. Therefore, discrepancies could not be generated by the extraction method and/or the preamplification step. One strain could not be recognized as RIF resistant by the MTBDR*plus* assay, thus suggesting that it may have a mutation outside the *rpoB* hot-spot region. Likewise, the false-INH-susceptible results may be explained by the fact that some INH resistance-associated mutations are not included in the MTBDR*plus* test, as previously reported (2, 8). It could be worth sequencing these strains to better understand the discrepancies between the MTBDR*plus* test and conventional DST results.

The S531L mutation in *rpoB* was most prevalent in the RIF^r isolates, at a frequency (30/47, 63.8%) similar to that generally reported elsewhere (1, 3, 5, 6, 8, 11, 13, 17). The most frequent INH^r mutations in this study were S315T in the *katG* gene (41/55 [75.56%]) and C15T in the *inhA* gene (12/55 [21.8%]), in keeping with results obtained by Hillemann et al. in 2007 in Germany (8) and Causse et al. in 2008 in Spain (5). Seven strains had both the wild-type pattern and mutant probe positivity (Table 2 and Table 3), possibly indicating the presence of a mixture of wild-type and mutated DNA sequences.

Although DNA extracted from slides has already been successfully used for sequencing (19, 24), the assays used in the latter studies are not suitable for routine detection of drug resistance in low-income countries.

To our knowledge, this is the first study to show the feasibility of using DNA extracted from stained slides for *M. tuberculosis* resistance testing with the MTBDR*plus* assay. The availability of stained smears in routine diagnostic laboratories and their easy transport and storage at room temperature mean that this approach is suitable for analysis of stained sputum smears collected in remote locations for the detection of MDR far more rapidly and sensitively than with conventional methods. Even resistance to second-line drugs like fluoroquinolones and injectable drugs should be detectable. Rapid detection of drug resistance nationwide would help not only to optimize treatment of MDR-TB but also to conduct resistance surveys aimed at identifying hot-spot regions of drug resistance

TABLE 3. Gene mutations conferring INH resistance identified by the MTBDR*plus* assay

Conventional DST[b] (no. of slides)	No. (%) of slides	MTBDR*plus* assay[a]			
		INH pattern (*katG*)	INH pattern (*inhA*)	Mutation or mutated codon (*katG*)	Mutation or mutated codon (*inhA*)
INH^r (55)	39 (66.1)	ΔWT, MUT1	WT	S315T	WT
	1 (1.69)	WT, MUT1	WT	S315T	WT
	3 (5.08)	ΔWT	WT	315	WT
	11 (18.64)	WT	ΔWT1, MUT1	WT	C15T
	1 (1.69)	ΔWT, MUT1	ΔWT1, MUT1	S315T	C15T
INH^s (4)	1 (1.69)	WT	ΔWT1, MUT1	WT	C15T
	1 (1.69)	WT, MUT1	WT	S315T	WT
	2 (3.39)	ΔWT1, MUT1	WT	S315T	WT

[a] WT, wild-type pattern; Δ, missing bands; MUT, mutation.
[b] INH^r, isoniazid resistant; INH^s, isoniazid susceptible.

in order to interrupt transmission chains. The feasibility and cost-effectiveness of using DNA extracted from stained sputum smears for the detection of anti-TB drug resistance in remote locations remain to be determined.

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REFERENCES

1. **Akpaka, P. E., S. Baboolal, D. Clarke, L. Francis, and N. Rastogi.** 2008. Evaluation of methods for rapid detection of resistance to isoniazid and rifampin in Mycobacterium tuberculosis isolates collected in the Caribbean. J. Clin. Microbiol. **46:**3426–3428.
2. **Anek-Vorapong, R., et al.** 2010. Validation of the GenoType MTBDRplus assay for detection of MDR-TB in a public health laboratory in Thailand. BMC Infect. Dis. **10:**123.
3. **Barnard, M., H. Albert, G. Coetzee, R. O'Brien, and M. E. Bosman.** 2008. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am. J. Respir. Crit. Care Med. **177:**787–792.
4. **Canetti, G., N. Rist, and J. Grosset.** 1963. Measurement of sensitivity of the tuberculous bacillus to antibacillary drugs by the method of proportions. Methodology, resistance criteria, results and interpretation. Rev. Tuberc. Pneumol. **27:**217–272. (In French.)
5. **Causse, M., P. Ruiz, J. B. Gutierrez, J. Zerolo, and M. Casal.** 2008. Evaluation of new GenoType MTBDRplus for detection of resistance in cultures and direct specimens of Mycobacterium tuberculosis. Int. J. Tuberc. Lung Dis. **12:**1456–1460.
6. **Evans, J., M. C. Stead, M. P. Nicol, and H. Segal.** 2009. Rapid genotypic assays to identify drug-resistant Mycobacterium tuberculosis in South Africa. J. Antimicrob. Chemother. **63:**11–16.
7. **Gori, A., et al.** 2005. Spoligotyping and Mycobacterium tuberculosis. Emerg. Infect. Dis. **11:**1242–1248.
8. **Hillemann, D., S. Rusch-Gerdes, and E. Richter.** 2007. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of Mycobacterium tuberculosis strains and clinical specimens. J. Clin. Microbiol. **45:**2635–2640.
9. **Hillemann, D., S. Rusch-Gerdes, and E. Richter.** 2009. Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of Mycobacterium tuberculosis strains and clinical specimens. J. Clin. Microbiol. **47:**1767–1772.
10. **Huang, W. L., H. Y. Chen, Y. M. Kuo, and R. Jou.** 2009. Performance assessment of the GenoType MTBDRplus test and DNA sequencing in detection of multidrug-resistant Mycobacterium tuberculosis. J. Clin. Microbiol. **47:**2520–2524.
11. **Huyen, M. N., et al.** 2010. Validation of the GenoType MTBDRplus assay for diagnosis of multidrug resistant tuberculosis in South Vietnam. BMC Infect. Dis. **10:**149.
12. **Kubica, G. P., W. E. Dye, M. L. Cohn, and G. Middlebrook.** 1963. Sputum digestion and decontamination with *N*-acetyl-L-cysteine-sodium hydroxide for culture of mycobacteria. Am. Rev. Respir. Dis. **87:**775–779.
13. **Lacoma, A., et al.** 2008. GenoType MTBDRplus assay for molecular detection of rifampin and isoniazid resistance in Mycobacterium tuberculosis strains and clinical samples. J. Clin. Microbiol. **46:**3660–3667.
14. **Minime-Lingoupou, F., et al.** 2010. Rapid identification of multidrug-resistant tuberculosis isolates in treatment failure or relapse patients in Bangui, Central African Republic. Int. J. Tuberc. Lung Dis. **14:**782–785.
15. **Miotto, P., et al.** 2009. Molecular detection of rifampin and isoniazid resistance to guide chronic TB patient management in Burkina Faso. BMC Infect. Dis. **9:**142.
16. **Mitchison, D. A.** 1984. Drug resistance in mycobacteria. Br. Med. Bull. **40:**84–90.
17. **Nikolayevskyy, V., et al.** 2009. Performance of the Genotype MTBDRPlus assay in the diagnosis of tuberculosis and drug resistance in Samara, Russian Federation. BMC Clin. Pathol. **9:**2.
18. **Rieder, H. L., et al.** 2008. Examen microscopique des frottis de crachats, p. 14–33. *In* Priorités pour les Services de Bactériologie de la Tuberculose dans les Pays à Faibles Revenus. International Union against Tuberculosis and Lung Disease, Paris, France.
19. **Silva, M. R., et al.** 2011. Identification of Mycobacterium tuberculosis complex based on amplification and sequencing of the oxyR pseudogene from stored Ziehl Neelsen-stained sputum smears in Brazil. Mem. Inst. Oswaldo Cruz **106:**9–15.
20. **Somoskovi, A., L. M. Parsons, and M. Salfinger.** 2001. The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in Mycobacterium tuberculosis. Respir. Res. **2:**164–168.
21. **Suresh, N., J. Arora, H. Pant, T. Rana, and U. B. Singh.** 2007. Spoligotyping of Mycobacterium tuberculosis DNA from archival Ziehl-Neelsen-stained sputum smears. J. Microbiol. Methods **68:**291–295.
22. **Suresh, N., et al.** 2007. Rapid detection of rifampin-resistant Mycobacterium tuberculosis directly from stained sputum smears using single-tube nested polymerase chain reaction deoxyribonucleic acid sequencing. Diagn. Microbiol. Infect. Dis. **58:**217–222.
23. **van der Zanden, A. G., et al.** 1998. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis complex in paraffin wax embedded tissues and in stained microscopic preparations. Mol. Pathol. **51:**209–214.
24. **Van Der Zanden, A. G., E. M. Te Koppele-Vije, N. Vijaya Bhanu, D. Van Soolingen, and L. M. Schouls.** 2003. Use of DNA extracts from Ziehl-Neelsen-stained slides for molecular detection of rifampin resistance and spoligotyping of Mycobacterium tuberculosis. J. Clin. Microbiol. **41:**1101–1108.
25. **World Health Organization.** 2008. Policy statement: molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). World Health Organization, Geneva, Switzerland.